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Salivary Syndrome in Horses: Identification of Slaframine in Red Clover Hay†

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An outbreak of salivary syndrome in horses in North Carolina was investigated. *Rhizoctonia leguminicola* was the predominant fungus isolated from toxic red clover hay. The fungus was less prevalent in the hay after 10 months of storage, and the hay had also decreased in biological activity after 10 months. Toxic hay caused extreme salivation, piloerection, lacrimation, respiratory distress, and increased frequency of defecation when fed to guinea pigs, and purified extracts of toxic hay and pure slaframine elicited these same responses when injected intraperitoneally into guinea pigs. The freshly acquired hay, based on the biological (slobber-producing) activity in hay and in purified extracts, contained the equivalent to 50 to 100 ppm (50 to 100 μg/g) of slaframine, but this level had decreased after 10 months by about 10-fold to about 7 ppm. Slaframine and seven synthetic derivatives of slaframine were used in the presumptive gas-liquid chromatographic identification of this mycotoxin. Slaframine (1-acetoxy-6-amino-octahydroindolizine) was identified in purified extracts of toxic hay by gas-liquid chromatography-mass spectrometry after preparative thin-layer chromatography. This was the first direct identification of slaframine in toxic red clover hay.

Slobbers (salivary syndrome) in cattle and horses is a mycotoxicosis which is most prominently characterized by excessive salivation. This syndrome in livestock has been reported by agricultural scientists since the late 1940s (16, 17) and was documented by O'Dell et al. (14), who demonstrated that the toxic hay and water or alcohol extracts of the toxic hay also caused slobbering in guinea pigs. Diarrhea, piloerection, excessive lacrimation, respiratory failure, feed refusal, and abortions are other symptoms reported in intoxicated animals, and in very severe cases, death can occur (4, 7, 14-17). Slobbers occur when livestock consume second-cutting legume hay or forage infected with the asporogenous fungus causing blackpatch on red clover (*Trifolium pratense L.*) and white clover (*Trifolium repens L.*), *Rhizoctonia leguminicola* Gough and Elliot (1, 4, 6, 10, 14-18). More infrequently, slobbering has been associated with forage grasses in the absence of red clover (P. B. Hamilton, N.C. State University, personal communication). White clover, soybean, kudzu, cow pea, blue lupine, alsike clover, alfalfa, Korean lespedeza, and black medic have also been implicated and are susceptible to infection by *R. leguminicola* (1, 4, 6, 10, 14-21). Blackpatch and salivary syndrome have been widely reported in the northwestern, midwestern, and southeastern United States, and the fungus was recently reported in Canada as well (1, 6, 10, 13, 15, 16, 19, 20).

All isolates of *R. leguminicola* tested produced a parasympathomimetic alkaloid, slaframine (1-acetoxy-6-amino-octahydroindolizine) (Fig. 1), in pure culture on red clover infusion or the antibiotic fermentation medium described by Gregory et al. (4, 11, 16, 17). Slaframine is also very toxic: the acute oral 50% lethal dose is 0.6 to 0.8 mg/kg of body weight in guinea pigs (4, 5, 7, 15-17), and doses of 0.1 to 0.3 mg/kg are reported to give a strong response in guinea pigs.

In previous research, parasympathomimetic activity in toxic hay or forage has been demonstrated by guinea pig bioassay, and subsequent physiological and characterization work was done with toxin preparations from cultures.

A serious outbreak of slobbers occurred in a herd of horses in the fall of 1979 near High Point, N.C. The intoxication was caused by a shipment of apparently high-quality second-cutting red clover-orchard grass hay from a usual supplier in West Virginia. Because cases of slobbers in horses are not as well documented as those in cattle and symptoms were very severe, samples of the toxic hay were brought to the Mycotoxin Laboratory at North Carolina State University

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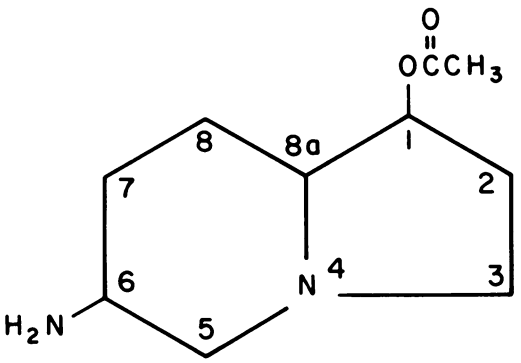


FIG. 1. *Structure of slaframine (1-acetoxy-6-amino-octahydroindolizine) proposed by Gardiner et al. (8).*

for examination. As a result of this work, we report, for the first time, the determination of slaframine directly in toxic hay.

(A preliminary report on a portion of this work was presented at the Annual Meeting of the American Phytopathological Society, Minneapolis, Minn., August 1980.)

MATERIALS AND METHODS

Isolation of *R. leguminicola* from toxic red clover. Red clover stems and leaves or bits of orchard grass were placed on water agar and incubated at 25°C for 24 to 48 h. Plates were then examined for the characteristic mycelium of *R. leguminicola* (1, 6, 17). Clover leaves were softened with sterile water and examined directly under the microscope for the presence of the fungus.

Isolation was repeated monthly for 10 months to examine the persistence of the fungus in the red clover. Bales of hay were stored indoors at room temperature, and clover from various points in the bales was examined for viable *R. leguminicola*. The fungus, once isolated, was maintained on clover infusion agar slants (7), potato dextrose agar slants, moist autoclaved rice, and moist autoclaved soil.

Animal tests. Suspect red clover was finely ground in a Waring blender and mixed at 50% (vol/vol) with Purina Guinea Pig Chow, which had also been finely ground. An orchard grass-Guinea Pig Chow diet was prepared in the same fashion, and the control diet of Guinea Pig Chow was also finely ground. Guinea pigs were housed, segregated by sex, on litter in two groups of four at 23°C. Test diets, control diets, and water were given ad libitum. Mature guinea pigs were fed test diets in groups of four or injected singly with test preparations. Guinea pigs were observed twice daily for 10 days for symptoms of salivary syndrome. The test was conducted for 10 days essentially as that of O'Dell et al. (14) as modified by Rainey et al. (15), Crump et al. (7), and Aust and Broquist (5).

Purified extracts of red clover and standards of authentic slaframine were prepared and injected intraperitoneally into guinea pigs also. Guinea pigs in this bioassay were examined for symptoms each 0.5 h for 2 h. No symptoms in 2 h was considered a negative test. Materials were prepared for injection by evaporation of chloroform under a stream of N_2, dissolution of residue in 1 ml of sterile water or physiological saline, and adjustment of the pH to 7 with sterile 0.1 N HCl. Injection volumes were 1 ml and contained 0.3 mg of pure slaframine per kg or the extract-equivalent of usually 50 or 100 g of dry red clover. Responses to injection were rated as: no response, mild, or strong.

Preparation of extracts and slaframine. Pure, authentic slaframine was prepared from slaframine dipicrate (14 mg) (a gift from H. P. Broquist, Vanderbilt University) by adjusting the pH to 9.5 with 0.1 N NaOH and extracting the free base into chloroform. Slaframine was stored in chloroform at −10°C, and samples were removed as needed.

Red clover or *R. leguminicola* mycelium was finely ground and extracted for 24 h in a Soxhelet extractor with 95% ethanol. Purification was essentially as described by Aust (4). Extracts in acidic solution were partitioned against $CHCl_3$, after which pH was adjusted to 10 and slaframine was extracted into chloroform. Extracts were stored at −10°C in chloroform, and the dipicrate was not made.

TLC. Thin-layer chromatography (TLC) was used in slaframine analysis. Samples of purified extracts were spotted on silica gel 60 TLC plates (0.25 mm by 20 cm by 20 cm) (E. Merck, Darmstadt, West Germany). Authentic slaframine was used for chromatographic standard. The solvent system (5, 15) was chloroform-methanol-ammonium hydroxide (90:10:1, vol/vol/vol). Plates or portions of plates were sprayed with Dragendorff's reagent (13) to make tertiary amines visible.

In preparative TLC, portions of extracts were streaked onto plates, and the plates were developed with the same solvent system. A band of silica gel at the R_f value of slaframine was scraped from the plate, and the silica was well crushed. Compounds were eluted from the silica gel with acetone. Eluates were concentrated and redissolved in chloroform for storage, guinea pig bioassay, gas-liquid chromatography (GLC), and GLC-mass spectrometry (MS).

Derivatives of slaframine. To determine whether mass spectral analysis of extracts for slaframine was indicated, analysis by GLC was conducted.

Derivatives of slaframine for GLC were prepared on a microgram basis by two different synthetic routes which led to *N*-acetyl-*O*-trimethylsilyl-deacetylslaframine (TMS-*N*-acetyl-*O*-deacetylslaframine). Derivatization of slaframine to *N*-TMS-slaframine (TMS-slaframine) and *N*-heptafluorobutyrylslaframine (HFB-slaframine) was also done. Reactions and extractions were carried out in 1/2-dram (ca. 1.8 ml) screw-cap vials in which the caps had been lined with polyethylene. Samples of active extracts of red clover or *R. leguminicola* cultures were derivatized in the same fashion for GLC analysis. Acetic anhydride and pyridine were reagent grade, and TriSil BT, heptafluorobutyric anhydride, and heptafluorobutyryl-imidazole were used as purchased (Pierce Chemical Co., Rockford, Ill.).

In the first route, slaframine was hydrolyzed at pH 12 with aqueous NaOH to *O*-deacetylslaframine, which was then extracted into $CHCl_3$. The compound

was then subjected to GLC analysis directly, reacted with TriSil BT to give *N*-TMS-*O*-TMS-deacetylslaframine (TMS-deacetylslaframine), or acetylated with acetic anhydride and pyridine to form *N*-acetylslaframine. *N*-acetylslaframine was then hydrolyzed with NaOH at pH 12 to *N*-acetyl-*O*-deacetylslaframine. This compound was then reacted with TriSil BT to give TMS-*N*-acetyl-*O*-deacetylslaframine. All of these derivatives were subjected to GLC analysis.

In the second synthetic route, slaframine was reacted with acetic anhydride and pyridine to give *N*-acetylslaframine. *N*-acetylslaframine was hydrolyzed with NaOH to give *N*-acetyl-*O*-deacetylslaframine, which was then extracted into $CHCl_3$ and reacted with TriSil BT to form TMS-*N*-acetyl-*O*-deacetylslaframine. These compounds were also subjected to GLC analysis.

In analogous reactions, slaframine was reacted with heptafluorobutyric anhydride and pyridine or heptafluorobutyryl-imidazole and subjected to GLC analysis.

GLC. GLC was conducted with a Shimadzu model GC-6AM custom gas chromatograph equipped with dual differential flame ionization detectors (Shimadzu Scientific Instruments, Columbia, Md.). The nickel column (1 by 3.2 mm) was packed with 1.7 g of 3% OV-17 on 100- to 120-mesh GasChromQ (Applied Science, Riviera Beach, Fla.). Oven programming was from 100 to 200°C at 8°/min. Carrier gas was N_2 at 40 ml/min, and H_2 and air for the flame ionization detectors were supplied at 100 ml/min. Detector response was recorded, and peaks were integrated with a Shimadzu CR-1A Chromatopak.

Purified red clover extracts, before and after preparative TLC, were derivatized in the same fashion, but amounts of extracts were used which should contain at least 0.5 μg of slaframine per μl injected into the gas chromatograph. This was based on the strength of the salivary responses in guinea pigs relative to the responses elicited by injection of known amounts of pure slaframine. A strong response was induced by injection of 0.3 to 0.5 mg/kg of body weight of pure slaframine.

GLC-MS. Mass spectra were obtained on a VG-Micromass, Ltd. model 7070F with Datasystem 2050 (Altrincham, England). Operating conditions were as follows: electron impact ionization at 70 eV, and scan speed was 1 s/decade (450-20). Spectra and chromatograms were plotted on the Versagel 800A, the mass spectrometer inlet line was at 200°C, the jet separator was at 230°C, and the ion source was operated at 210°C.

The mass spectrometer was equipped with a Hewlett-Packard 5710A gas chromatograph (Palo Alto, Calif.) fitted with a 2-m glass column packed with 3% OV-17 on a solid support of 100- to 120-mesh GasChromQ (Applied Science). The oven was programmed from 130 to 250°C at 8°C/min, helium was the carrier gas at a flow rate of 20 ml/min, and the injector was operated at 200°C. The TMS derivative of slaframine was selected for GLC-MS analysis.

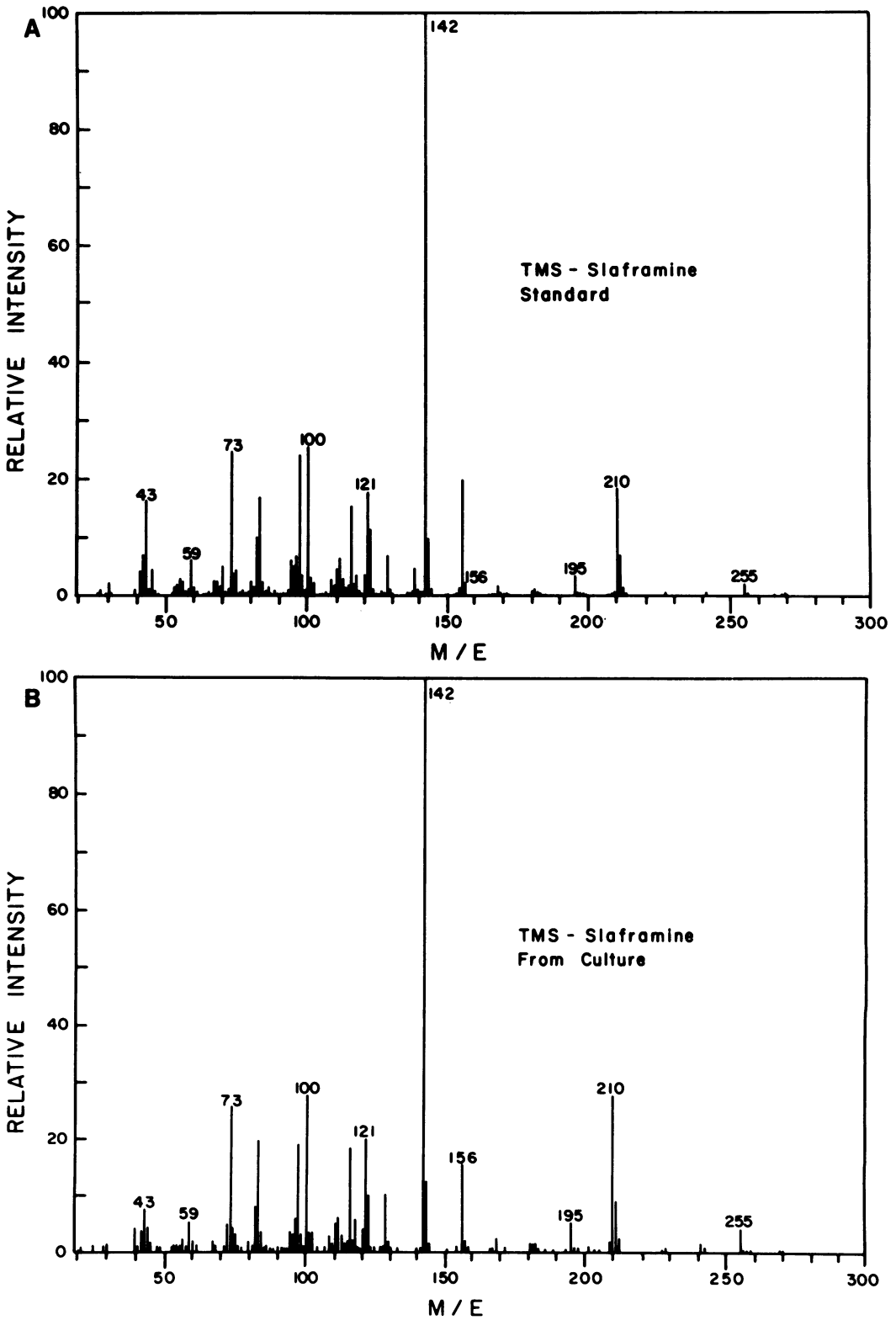
Production of slaframine by *R. leguminicola* isolated from toxic red clover. Fresh red clover (500 g) was mixed in a Waring blender with 1 liter of distilled water. The mixture was filtered through cheesecloth and diluted to a 2-liter volume with distilled water. The extract was divided among 2.8-liter Fernbach flasks (200 ml each), and the flasks were stoppered with cotton plugs and autoclaved at 121°C for 0.5 h. When the clover extract medium had cooled, flasks were seeded with a rice culture of *R. leguminicola*. Incubation was at 25°C for 21 days. After the incubation period, mycelial mats were collected, blotted dry, and extracted with 95% ethanol in a Soxhelet apparatus. Culture media were discarded. Slaframine was found to be retained in the thallus and not released into the medium (16, 17). The extract was purified as described by Aust (4), and the purified extract was analyzed by TLC, GLC, and GLC-MS. Slaframine in extracts of *R. leguminicola* was identified by its mass spectrum.

RESULTS AND DISCUSSION

R. leguminicola was isolated from all of the samples of red clover tested in the first month. It was recognized by its dark (black or dark green) asporogenous mycelium, which grew rapidly over the plate. Mycelial characteristics (color and morphology) were as described (1, 4–6, 10, 16–18, 20, 21). In fact, the characteristically branching mycelium of the fungus could be seen on direct microscopic examination of the clover leaves. The fungus and slobber-producing activity persisted over a 10-month period, but both grew less prevalent with time (data not shown). The fungus was never isolated from orchard grass hay.

Orchard grass, on feeding, caused only one animal to salivate. This was probably because of contamination with red clover due to the difficulty of complete separation (Table 1). The red clover itself, when fed, and purified extracts of red clover, when injected, caused extreme salivation and lacrimation in guinea pigs. One of the animals fed toxic hay died of respiratory failure. None of the injected guinea pigs died. The purified extracts caused slobbering, lacrimation, diarrhea, etc., but these symptoms were not of as long duration as those caused by feeding the toxic red clover, nor were the injected animals as lethargic or ill. This suggested that the extraction and purification of toxin were inefficient or other toxins with similar activity were present in the hay. It is noteworthy that other researchers also suggested that slaframine's activity could not account for all of the symptoms observed in field outbreaks of slobbers (16, 17). The 10-day feeding test is necessary because the guinea pigs may refuse to eat for several days after the first meal.

Slaframine standards and extracts of the hay were analyzed by TLC and GLC. Its retention time in GLC is shown in Table 2. After TLC the chromatogram was still too complex for definitive analysis, but after preparative TLC and


A
B
RELATIVE INTENSITY
M / E
TMS - Slaframine
Standard
TMS - Slaframine
From Culture
142
73
100
121
43
59
156
195
210
255
0
20
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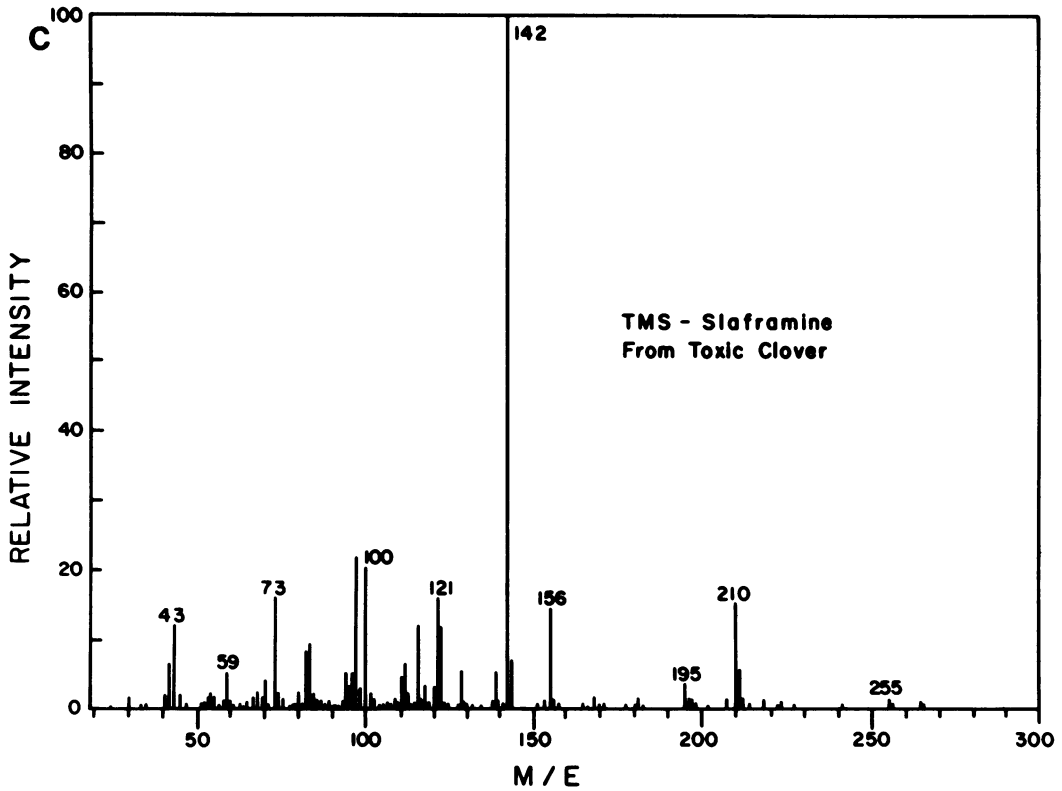

FIG. 2. *Mass spectra of TMS-slaframine. (A) TMS-slaframine standard. (B) TMS-slaframine from R. leguminicola culture. (C) TMS-slaframine from toxic red clover.*

TABLE 1. *Guinea pig bioassay for slaframine*

Treatment	Response[a]
Feed	
Control feed	−,−,−,−
Clover: control feed (1:1, vol/vol)	+,+,+,+[b]
Orchard grass: control feed (1:1, vol/vol)	−,−,−,+
Intraperitoneal injection	
Injected, control (physiological saline)	−,−,−,−
Injected, clover extract (100 g equivalents)	+,+,+,+
Injected, slaframine (0.3 mg)	+,+

[a] Each + or − represents the response of one animal. A positive response is salivation, piloerection, lacrimation, and diarrhea.

[b] One test animal died of respiratory failure.

TABLE 2. *GLC of slaframine and some derivatives of slaframine*[a]

Compound	Retention time (min)
Slaframine	5.3
O-Deacetylslaframine	3.4
TMS-slaframine	6.0
TMS-*O*-deacetyl-*N*-acetylslaframine	9.6
N-Acetylslaframine	11.4
N-Acetyl-*O*-deacetylslaframine	10.0
TMS-*O*-deacetylslaframine	3.9
HFB-slaframine	13.5

[a] Shimadzu GC-6AM chromatograph, 3% OV-17 on 100- to 120-mesh GasChromQ, N_2 carrier gas, flame ionization detection, temperature programmed from 100 to 200°C at 8°/min.

GLC the gas-liquid chromatogram was clear and easy to interpret. TLC analysis of purified extracts was inconclusive because several components of red clover hay had R_f values similar to those of slaframine and were positive to Dragendorff's reagent.

Derivatives of pure slaframine were prepared for use as standards for GLC analysis of the toxic extracts; there were no chromatographic differences between derivatives from the different synthetic pathways used nor were there chromatographic differences in HFB-slaframine preparations from heptafluorobutyryl-imidazole or heptafluorobutyric anhydride (Table 2). Although the structures of these derivatives were not proven, all of these reactions and derivatizations of slaframine were, with the exception of the reaction with TriSil BT, reported by at least two different research groups when characterization and proof of structure of slaframine were progressing (2–5, 7–9, 14, 15, 18).

Samples of the hay extracts were derivatized and analyzed by GLC. Components in extracts

were observed which corresponded to the slaframine derivatives. Slaframine, deacetylslaframine, *N*-acetylslaframine, *N*-acetyl-*O*-deacetylslaframine, and TMS derivatives of slaframine, deacetylslaframine, and *N*-acetyl-*O*-deacetylslaframine were used in the GLC analyses to give presumptive identification of slaframine in the toxic hay (Table 2). Even though GLC of pure slaframine is possible, column life was extended by derivatization of clover extracts, and TMS derivatives were also an aid in mass spectral interpretation because of the increase in molecular weight obtained and the characteristic fragmentation patterns obtained with these derivatives.

Preparative TLC followed by GLC-MS analysis allowed achievement of an isolated slaframine peak and a full mass spectrum of TMS-slaframine in an extract of toxic clover (Fig. 2). This was the first direct identification of slaframine in toxic clover. Mass spectra of a slaframine standard, slaframine from toxic clover, and slaframine from cultures of *R. leguminicola* are shown in Fig. 2. They exhibited identical fragmentation patterns.

The mass spectra of the TMS-slaframine standard, as well as those of TMS-slaframine in toxic clover extracts and of TMS-slaframine from *R. leguminicola* cultures, were identical (Fig. 2). The molecular ion (M^+) of TMS-slaframine was 270 and was of very low intensity in the spectrum. Prominent fragments at m^+/e = 255, 210, and 195 represented losses of M^+-15,CH_3-; M^+-60,CH_3COOH; M^+-75,CH_3- and CH_3COOH, respectively, from the molecule. It is very interesting to note that mass spectral evidence indicates that probably only one TMS group adds to the amine function at C-6 of slaframine. Mass spectra of the other derivatives were not made, but this chemistry has been reported (2–4, 5, 7–9, 14, 15). Mass spectra of the pure slaframine standard (not shown) were identical and similar in all respects to mass spectral information presented by Aust (4) and Aust and Broquist (5).

Although efficiency of recovery from toxic hay is unknown, quantification of slobber-producing activity by response produced in the guinea pig test relative to that produced by known amounts of pure slaframine gave estimates of slobber-producing activity in the clover ranging from the equivalents of 50 to 100 ppm (50 to 100 μg/g) of slaframine per g (dry weight) of clover. After 10 months of storage, the activity of the hay had decreased to the equivalent of about 7 ppm based on response in the guinea pig bioassay.

A slaframine-producing isolate of *R. leguminicola* was isolated from toxic red clover hay and the mycotoxin slaframine was identified per se for the first time. GLC-MS was used for the identification of the slaframine in toxic red clover. Thus, Koch's postulates as applied to mycotoxins have been satisfied (12), and the conclusions drawn from the earlier work which implicated slaframine as the cause of this mycotoxicosis would seem to be confirmed.

Although blackpatch occurs only sporadically, it can cause serious economic loss to the cultivators of red clover and those who attempt to graze or feed toxin-laden clover. In fact, it has forced many farmers to grow other legumes which are less susceptible or not susceptible to the fungus.

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